Engineering biofilms for biocatalysis

Journal Item

Biofilm: Friend Not Foe
Engineering Biofilms for Biocatalysis

Andreas N. Tsoligkas, Michael Winn, James Bowen, Tim W. Overton, Mark J. H. Simmons*, Rebecca J. M. Goss*

There is an increasing trend of using biotransformations in the fine chemicals industry[1,2], however a key problem facing this technology is the sensitivity of enzymes and even whole cells to extremes in pH, temperature and mechanical stress. Although there has been significant research into using extremophiles, fragility is still an issue[3]. A solution may be found by employing biofilms, communities of cells protected by a secreted matrix; these are robust structures capable of withstanding physical and chemical extremes.

In their natural environment, planktonic cells cluster together at surfaces and interfaces. Within these clusters they protect themselves from environmental and chemical stress by secreting extracellular polymeric substances (EPS), a protective and adhesive polysaccharide matrix[4]. Historically, biofilms are perceived as problematic, their robust structure being difficult to remove from surfaces and they are associated with infections, dental caries and marine and reactor fouling[5,6]. However, in the arena of biocatalysis there is scope to exploit biofilms and their ability to tolerate organic chemicals and physical extremes that planktonic microbes and purified enzymes cannot. So far the use of biofilms in biotransformations has been largely confined to the use of consortia of mixed bacterial species in wastewater treatment and bioremediation. Reports of the use of biofilms for synthesis are limited to simple compounds such as acetic acid, ethanol, butanol, 2,3-butanediol, lactic acid, fumaric acid, succinic acid and styrene oxide[7,8,9]. With the exception of the report of a recombinant strain into which D-amino acid oxidase activity was introduced [10], there are no accounts of the use of specialized recombinant strains as immobilized single species biocatalysts. We wished to develop an engineered immobilized biocatalyst into which any gene of choice could be introduced, as a plug-and-play platform, enabling the synthesis of a particular series of fine chemicals. We envisaged that such a biological catalyst would be a useful component for flow chemistry.

Current approaches to generating biofilms only incorporate a small percentage of the overall biomass. The engineered deposition of a biofilm is attractive as it enables the capture of a larger proportion of cells and the possibility to control microstructure. We determined that spin coating could be employed to control biofilm formation, and examined whether such artificially deposited films exhibited similar characteristics to natural biofilms. To test this approach *Escherichia coli* PHL644, which overproduces curli and readily forms biofilms[11], was transformed with pSTB7, a high copy number plasmid expressing tryptophan synthase from *Salmonella enterica* sv Typhimurium[12]. The transformants were grown in ½ LB broth supplemented with ampicillin. Once an OD_{600} of 2 was reached the cells were spin-coated onto a poly-L-lysine coated flat glass substrate using centrifugation (1851g, 10 mins). The slides were then matured in minimal M63 medium in an orbital shaker incubator (30°C at 70 rpm, throw of 19 mm) for up to 7 days prior to analysis or use in biotransformations (full details are reported in the Supporting Information (SI)). In parallel, an equivalent biomass of transformed *E. coli* was allowed to form natural biofilms under identical conditions but without employing the spin coating step. This was achieved by incubating cells suspended in M63 media with poly-L-lysine coated glass slides (30°C at 70 rpm, throw of 19 mm, 7 days). The topology and adhesive forces within the engineered and naturally deposited biofilms were explored using ESEM and AFM (Fig. 1) enabling high resolution and global analysis of the structure and strength of the biofilm. Using these tools we followed the maturation, growth and increase in stability of the engineered biofilm. After three days maturation of the engineered biofilm, shallow pores and channels could already be observed (see Supporting information, Fig. 4a). The most dramatic change in biofilm development was observed between days 5 and 6, with a sharp increase in the adhesive force as measured by AFM from 0.81 nN to 40 nN (Fig. 1a). The timing of this increase in strength corresponded to the observed production of the adhesive extracellular polymeric substance (EPS), visible by ESEM as a white fibrous material on the surface of the cells following 6 days of maturation (Fig. 1f, as indicated by arrow) but not after 5 days (Fig. 1e). This 'sticky' matrix is known to promote biofilm stability[13]. At day 6 and day 7, deep pores and channels were observed by ESEM (see Fig. 1f and SI Fig. 4e respectively), conferring a large catalytic surface area to the biofilm. Strikingly, whilst the ESEM image of the engineered biofilm following 6 days' maturation (Fig. 1f) shows almost complete surface coverage and a three dimensional growth morphology the natural biofilm, grown under the same conditions, is only present as a sparse monolayer following 7 days' growth (Fig. 1d).

Thickness and surface roughness of the biofilm were measured during the maturation period using interferometry (see Fig. 1b) and showed a notable increase between days 3 and 7, consistent with the growth of mushroom like structures during biofilm formation (see Fig. 1c). Between days 7 and 8, roughness decreased, consistent with the 'caps' of the mushroom structures fusing, forming a smooth surface on top of which further mushroom colonies formed in days 9 and 10, once more increasing roughness and thickness (up to 63 µm on day 10; Fig. 1c).

Before utilising the engineered biofilm as an immobilised catalyst, its stability in the reaction buffer was tested. Global stability tests were carried out by placing the engineered biofilm (matured for 7 days) into the reaction buffer. At intervals, 1 mL aliquots of reaction buffer were removed and the OD_{600} measured in order to determine the amount of planktonic cells that had left the biofilm (Fig. 2). The OD_{600} value representing 100% loss of biofilm from the slide was determined by completely re-suspending engineered biofilm in M63 medium (mean values as calculated from the re-suspension of >10 biofilms). For further experimental details see SI. Through these experiments we demonstrated that at least 90% of the biomass remained entrapped within the biofilm during a 24 hour period of incubation within the biotransformation reaction buffer. Following 40 hours of incubation, just under 80% of the cells remained entrapped. This represents a high level of cell entrapment and biofilm stability. Having determined our

[*] Dr. A. Tsoligkas, Dr. J. Bowen, Dr. T. W. Overton, Dr. M. Simmons
School of Chemical Engineering, Birmingham University,
Edgbaston, Birmingham, B15 2TT (UK)
E-mail: m.simmons@bham.ac.uk

Dr. R. J. M. Goss, M. Winn, School of Chemiistry, University of East Anglia, Norwich, NR4 7TJ (UK)
E-mail: r.goss@uea.ac.uk
Homepage:www.uea.ac.uk/cap/people/faculty/rjmg/

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engineered biofilm to be robust, we assessed its ability to mediate biotransformations. Within the context of these scoping studies we examined the enantioselective tryptophan synthase mediated conversion of haloindoles and serine to L-halotryptophans[12]. L-halotryptophans are a useful starting material for the synthesis of a series of bioactive natural products such as the anticancer agents rebeccamycin and diazonamide or for use in generating modified peptides and proteins.[14-20] The biocatalysis of serine plus 5-fluoro-, 5-chloro-, and 5-bromoindole to the corresponding halotryptophan using a cell free lysate containing tryptophan synthase under free and immobilised conditions has been reported[14,15], as has the use of purified tryptophan synthase in the conversion of 5-chloroindole to 5-chlorotryptophan.[16] (Conditions and conversion levels are detailed in Table 1)

For our assessment of the ability of the engineered biofilm to catalyse this biotransformation, engineered biofilms that had matured for 7 days were transferred to the reaction buffer; the reaction was initiated by the addition of indole analogue and incubated at 30°C for 30 hours in an orbital shaking incubator. Following the reaction, the yields for 5-fluoro and 5-chlorotryptophan production by the engineered biofilm were notably higher than those for the free or immobilized cell lysate (93% vs 63%/83% and 78% vs 50%/61% respectively: See Table 1). Conversions were calculated based on the concentration of halotryptophan product as assessed by an HPLC assay(see SI); the accuracy of this method was confirmed through correlation with the yield obtained by extracting a known quantity of halotryptophan from a sample and by extracting and purifying halotryptophans from a sample number of reactions.

We wished to determine whether general trend in increase in yield was due to the enhanced stability and longevity of active enzyme within the biofilm embedded cells. In order to gain a greater understanding of this, the course of the reaction was followed by the extraction of aliquots of reaction buffer, the concentration of product being determined by HPLC (see SI). The time course of the reaction for the engineered biofilm mediated conversions of 5-fluoro, 5-chloro and 5-bromoindole were compared. It is notable that at 30 hours the rate of conversion of 5-chloro and 5-bromoindole by the engineered biofilm was the same as at the start of the reaction, demonstrating that there had been no observable loss in catalytic activity (Fig. 3iia and iii).

The rate of conversion of the 5-fluoroindole was observed to decrease after 24 hours, this is likely to be due to the fact that at this point almost 90% of the starting material had been consumed.

The total biomass immobilized on each slide was determined to be 80 mg by analysis of total dry mass and by total protein analysis (see Supporting Information). To examine the performance of the tryptophan synthase within non-immobilized cells we carried out the biotransformation of the 5-chloroindole under the same reaction conditions using the equivalent of 160 mg dry weight cells (double the biomass present in the biofilm system and at least 2 x the specific protein content). These time courses indicate that though conversion of 5-chloroindole to 5-chlorotryptophan is initially much faster with the free cells (Fig. 3, iic; as one might expect due to increased surface area, ease of substrate uptake and potentially double the amount of catalyst) there is no observable catalytic activity after 24 hours.

The immobilisation of enzymes, for example by using a polyhistidine tag to enable binding to nickel-nitrilotriacetic (Ni-NTA) resin, has been shown oftentimes to improve catalytic activity; we sought to determine whether this would be the case for tryptophan synthase. Tryptophan synthase is a two subunit enzyme (normally found as a $\alpha_2\beta_2$ tetramer). We achieved immobilization of the β subunit (responsible for performing the biotransformation) through a *C*-terminal polyhistidine tag.

The α subunit (required for generation of indole *in vivo* and for complex stability) associates sufficiently to the β subunit to be co-purification and immobilisation to occur. Using the generous assumption that typtophan synthase made up 100% of the total protein mass in the biofilm, an equivalent amounts of this resin immobilised enzyme was used to catalyse the 5-chloroindole biotransformation. The initial rate of the immobilised enzyme (Fig. 3, iib) was far greater than the biofilm or the non-immobilised cells, as no barrier is present to reduce access to the enzyme. The reaction, however, ceases after only 5 hours. Analysis of the enzyme following the reaction (after 30 hours) reveals that all of the β-subunit and at least 40% of the α-subunit had remained immobilised.

To gain further insight into the improved longevity of the enzyme within the protective environment of biofilm embedded cells, we explored the possibility of recycling the engineered biofilm catalyst. From our experiments on the stability of the biofilm, we had demonstrated that more than 90% of cells remained entrapped over a 24 hour period. Working within this time frame we investigated the catalytic ability of the biofilm in three separate, sequential, 12 hour reactions (Fig. 4). During these sequential reactions the biofilm remained intact and no significant reduction in rate was observed.

The engineered biofilm shows considerable promise for the biotransformation of fine chemicals, with our results demonstrating that in the case of tryptophan synthase, the engineered biofilm is a significantly better catalyst than purified protein or the cell free lysate.[16,14] We believe that the engineered biofilm has great potential both for the industrial biotransformation of fine chemicals and for use as a component within flow chemistry. The forced deposition of the biofilm leads us to believe that there may be scope for engineering the microstructure of this biocatalyst.

Experimental Section

Full accounts of all experiments reported in this manuscript are detailed in the Supporting Information.

Biofilm preparation and maturation All samples in this study were investigated in the form of thin layers on glass microscope slides (75 mm by 25 mm, VMR). Prior to biofilm formation, glass slides were coated with approximately 4 mL of 0.1% (w/v) poly–L–lysine (PLL) in water (Sigma), which was then dried overnight in an oven at 60°C. 10 µL of a culture of *E. coli* PHL644 transformed with pSTB7 was streaked onto an agar plate supplemented with ampicillin and incubated at 37°C for 14 hours. Single colonies were picked and used to inoculate 200 mL of ½ LB supplemented with ampicillin (100 µg mL^{-1}). The culture was incubated in an orbital shaker at 30°C, 180 rpm with a throw of 19 mm for 16 hours.

Following incubation, cultures were transferred aseptically into sterile 750 mL polypropylene centrifuge bottles (Beckman Coulter UK Ltd.) containing the PLL-coated glass slides supported on a bed of glass beads (200 g, soda-glass beads, 4 mm diameter) to provide a flat surface to prevent cracking during centrifugation and were centrifuged at 1851g for 10 minutes in a centrifuge fitted with a swinging bucket rotor. After centrifugation, the glass slides were gently placed in 500 mL sterilised wide necked Erlenmyer flasks (Fisher Scientific) containing 70 mL of M63 medium supplemented with ampicillin (100 µg mL^{-1}). The spin coated biofilms were incubated in an orbital shaker incubator at 30 °C, 70 rpm with a throw of 19 mm (set at a low speed to minimise bacteria shearing from the biofilm) for a maturation period of 7 days. For comparison, natural biofilms were generated by harvesting the 16-hour cultures by centrifugation (1851g for 15 mins), and resuspending the bacteria in 70 mL of M63 medium in a 500 mL wide necked Erlenmyer flask into which the PLL coated glass slide was introduced. The slide was incubated for 7 days at 30°C and 150 rpm, in an orbital shaker incubator with a throw of 19 mm.

Biotransformation with Biofilm

Engineered biofilms that had matured for 7 days were transferred to the reaction buffer (0.1 M KH_2PO_4, 7 mM Serine, 0.1 mM PLP, adjusted to pH 7.0). The biofilm maturation M63 medium was carefully removed from the biofilm covered slide using a syringe. Un-adhered, planktonic cells were removed from the biofilm by gentle re-submersion and washing in aliquots of reaction buffer (2 x 50 mL), which was then also removed. The washed biofilm slide was then submerged in 70 mL of reaction buffer supplemented with 0.7 M DMSO and either 2 mM 5-chloroindole (0.0212 g), 2 mM 5-fluoroindone (0.0189 g), or 2 mM 5-bromoindole (0.0274g).

The biotransformation reactions were placed into an orbital shaker incubator (30°C, 70 rpm with a 19 mm throw), set at a low speed to minimise cell shearing, and incubated for 30 hours. 0.5 mL aliquots of the reaction buffer were taken every hour for the first 7 hours and then at regular intervals thereafter. Any reaction in the samples was stopped by centrifugation (16060g, 5 min) in order to remove any planktonic cells in solution. The concentration of 5-halotryptophan in each of the aliquots was determined by HPLC analysis.

Keywords: biocatalysis · enzymes · amino acids · immobilisation · flow chemistry

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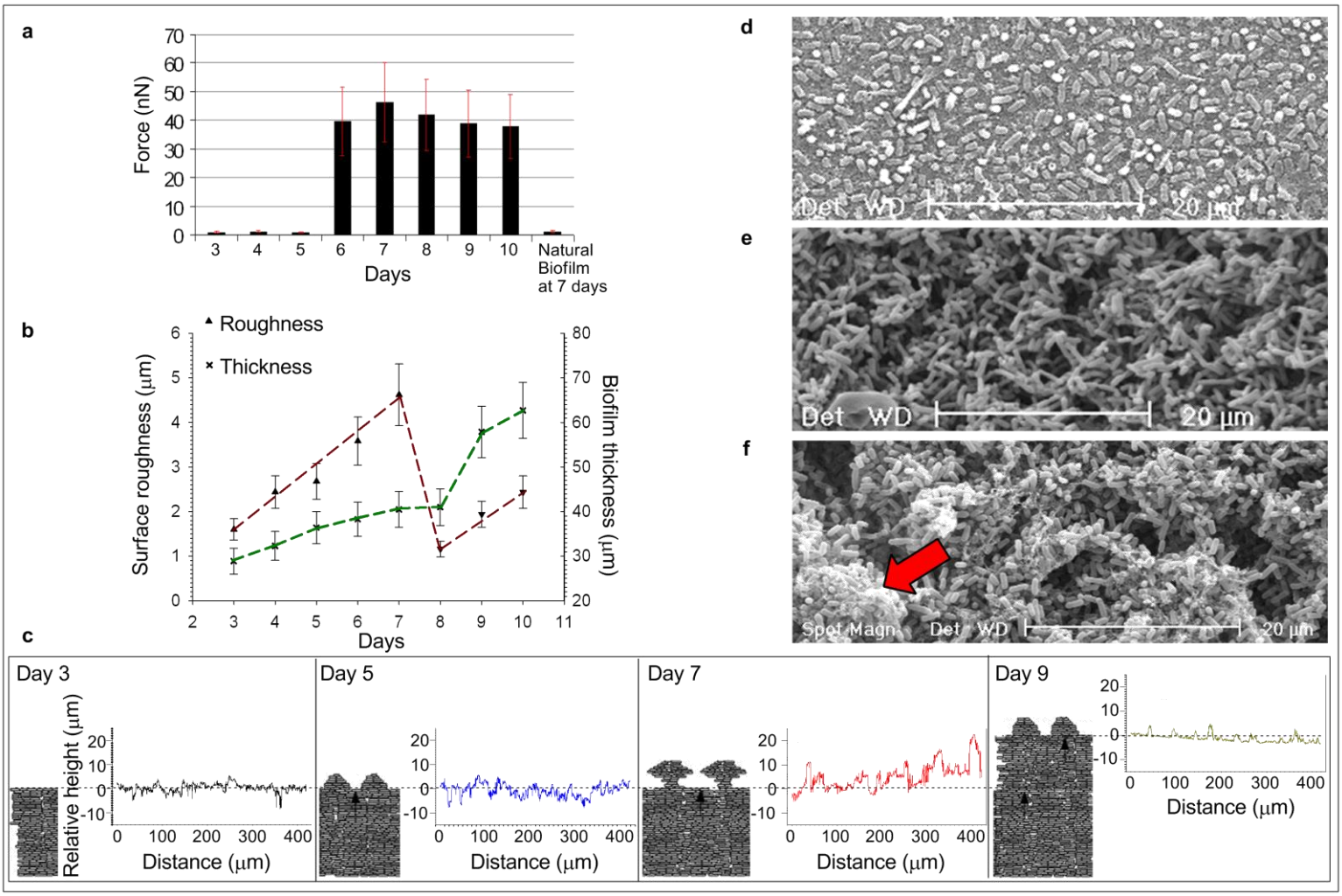

Figure 1. Surface structure and strength of engineered biofilm.
(**a**) AFM measurements of biofilm adhesive force, and (**b**) Variation of biofilm roughness and thickness, measured by interferometry, at different stages of maturation from 3 days to 10 days. (**c**) Changes in surface topography of the engineered biofilm at 3,5,7 and 9 days of maturation (determined using interferometry) with cartoons showing how the changes in surface structural features (mushroom colonies) affect the observed surface topography. Distance refers to the horizontal measurement across the biofilm. (**d**) ESEM image of a natural biofilm after 7 days of maturation. Only a sparse coverage can be observed (3880x magnification). (**e**) ESEM image of the engineered biofilm following 5 days maturation (3886 x magnification). (**f**) ESEM image of the engineered biofilm following 6 days maturation (3906 x magnification), the arrow indicates the presence of EPS. The formation and deepening of pores and channels within the biofilm may be observed.

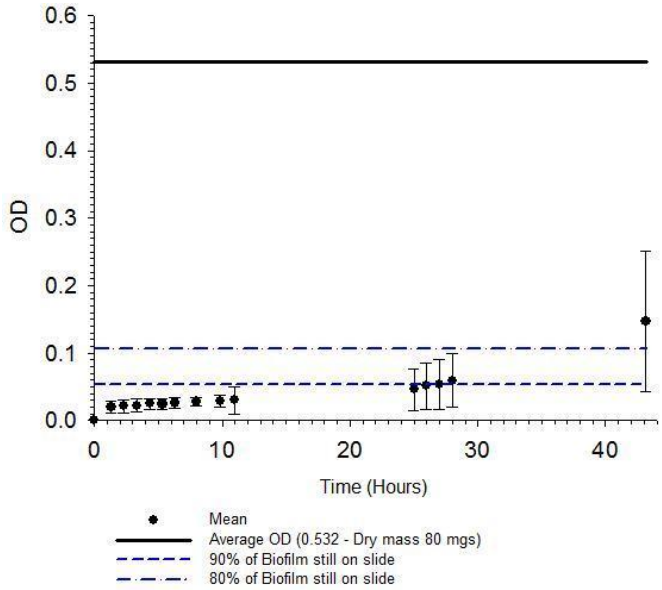

Figure 2. Stability of the engineered biofilm in reaction buffer. The number of cells sheared from the biofilm over 40 hours was determined by measuring the OD_{600} of the reaction buffer and comparing it to the OD_{600} of reaction buffer containing a fully re-suspended biofilm.

Table 1. Comparative yields of 5-halotryptophans generated using: a) purified enzyme, b) enzyme as component of cell free lysate, c) immobilised catalyst, d) engineered biofilm, e) planctonic cells

Tryptophan Generated	Catalyst: enzyme as component of cell free lysate	Catalyst: Ni-NTA resin Immobilised Enzyme	Catalyst Planktonic cells	Catalyst: Engineered Biofilm
Biotransformation Conditions	3 days, 37°C	30 hours, 37°C	30 hours, 30°C	30 hours, 30°C
Relative Protein Concentration	0.4 mg ml^{-1} (total protein content)	0.4-0.6 mg ml^{-1} (tryptophan synthase alone)	1.2 mg ml^{-1} (whole cell protein content)	0.6 mg ml^{-1} (total biofilm protein content)
	Conversion	Conversion	Conversion	Conversion
5-F	63% [14]			93%
5-Cl	50% [14]	49%*	40%**	78%***
5-Br	16% [14]			18%*

[*]no further reaction is observed after 6 hours [**]no further reaction is observed after 24 hours [***]reaction is still proceeding at initial rate following 30 hours (see Fig. 3 iia and and iii)

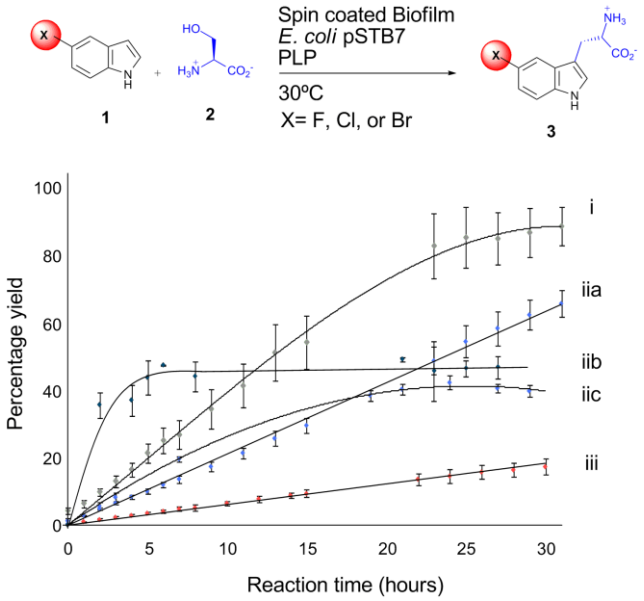

Figure 3. Example biotransformations with tryptophan synthase. Biotransformation of 5-haloindole (1) and serine (2) into 5-halotryptophan (3) reaction profile for (i) 5-fluoroindole (biofilm) (ii a) 5-chloroindole (biofilm), (ii b) 5-chloroindole (immobilised enzyme), (ii c) 5-chloroindole (incubated with twice the biomass of free cells) (iii) 5-bromoindole (biofilm).

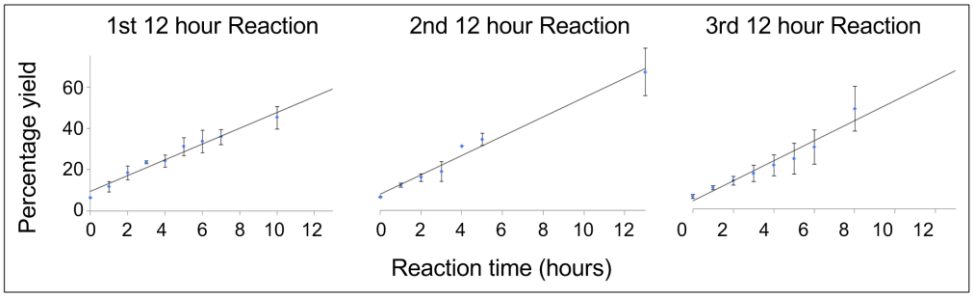

Figure 4. Recycling the biofilm leads to little observed loss in activity. 3 Sequential 12 hour 5-chloroindole biotransformation reactions with the same biofilm are illustrated.